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(54) Title: ENZYMATIC DETERGENT

(57) Abstract

Amino acid-specific proteases show excellent detergency, and the presence of non-specific protease decreases the detergency. An increasingly better performance is observed when preparations containing mixtures of proteases are purified to contain only specific proteases. Purified specific proteases are even observed to be superior to the unspecific proteases used in detergents today.

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ENZYMATIC DETERGENT

TECHNICAL FIELD

This invention relates to a protease-containing detergent composition and to a proteolytic detergent additive for use therein.

BACKGROUND ART

Proteases are widely used as ingredients in commercial detergents to improve the detergency towards proteinaceous soiling. Further information on this may be found in the article "How Enzymes got into Detergents", vol. 12, Developments in Industrial Microbiology, a publication of the Society for Industrial Microbiology, American Institute of Biological Sciences, Washington, D.C. 1971, by Claus Dambmann, Poul Holm, Villy Jensen and Mogens Hilmer Nielsen, and in P.N. Christensen, K. Thomsen and S. Branner: "Development of Detergent Enzymes", paper presented on 9 October 1986 at the 2nd World Conference on Detergents held in Montreaux, Switzerland.

As indicated in said references, trypsin preparations were previously used in detergents, but since the 1960'es alkaline Bacillus proteases have been used almost exclusively for this purpose, and in this period large efforts have been devoted to the development of microbial proteases with improved detergency.

It is known that whereas pure trypsin is very specific hydrolyzing only a few peptide bonds in any given protein, the commonly used Bacillus protease have a broad specificity and thus hydrolyzing many bonds in a given substrate.

In the search for improved proteases it has been assumed that such a protease should have the broadest possible substrate specificity, i.e. it should be able to hydrolyze as many bonds as possible in the protein soiling.

Thus, M. Minagawa, Osaka Shiritsu Daigaku Seikatsu Kagaku-bu Kiyo, vol. 23, pp. 65-74 (1975) states on p. 68: "The type of protease adapted for use in the removal of protein stains must have a wide spectrum of substrate specificity capable of degrading the peptide bond, indiscriminately."

STATEMENT OF THE INVENTION

Surprisingly, we have now found that amino acid-specific proteases show excellent detergency, and that the presence of non-specific protease decreases the detergency.

We have thus observed an increasingly better performance when preparations containing mixtures of proteases are purified to contain only specific proteases. We have even observed purified specific proteases to be superior to the unspecific proteases used in detergents today.

Commercial preparations of the specific protease trypsin, which contain varying amounts of other proteases, have been used in detergents but the use of essentially pure trypsin in detergents is novel.

Microbial specific proteases essentially free from other proteases are also known but their use in detergents is novel.

Our working hypothesis is that the improved detergency is due to the fact, that large peptide fragments are removed more efficiently during washing conditions. This may also explain why commercial preparations of trypsin, which contain varying amounts of other proteases have never been considered superior to the unspecific Bacillus proteases used in detergents today.

Accordingly, the first aspect of the invention provides a detergent composition comprising protease, characterized in that the protease activity affecting detergency is essentially provided by a protease with specificity for one or two amino acids.

The second aspect of the invention provides a detergent additive comprising protease in the form of a non-dusting granulate, a stabilized liquid or a protected enzyme, characterized as above.

DETAILED EXPLANATION OF THE INVENTION

Amino acid-specific protease

The class of proteases that may be used in the present invention are those endoproteases (endopeptidases) that hydrolyze proteins by preferentially cleaving peptide bonds adjacent to one or two amino acids giving rise to large peptide fragments. A number of such proteases are known, especially of animal and microbial origin. See K. Morihara: "Comparative Specificity of Microbial Proteinases", Adv. Enzymol. Relat. Areas Mol. Biol., 41, 179-243 (1974).

The specificity may be for bonds on the amino or carboxyl side of one or two specific amino acids. More particularly, the protease may have trypsin-like specificity, and it may be trypsin or microbial, trypsin-like protease.

The protease may be of serine, thiol, metal or aspartate type.

Recombinant DNA technology may be used to provide a microorganism containing a gene that encodes for and expresses and preferably also excretes the specific protease. This organism may be cultivated to produce protease for use in the invention.

Preferred specific proteases for use in the invention are active in the pH range 6-12, especially 7-10.5, and most preferably have pH optimum in either of these pH ranges.

The preparations of the invention are preferably essentially devoid of other proteases. Typically, specific protease (as defined above) makes up more than 90% and especially more than 95% of the total protease present, on weight basis or activity basis (e.g. measured in CPU, described below).

Usually, the specific protease activity will be provided essentially by a single specific protease. But a mixture of two (or more) specific proteases may be used, provided the mixture shows the stated specificity, e.g. a mixture of proteases with the same specificity.

One particularly preferred protease is a novel Fusarium protease discovered by the inventors and described below.

Novel Fusarium protease

The novel protease was isolated from a microbial strain which has been deposited on 6 June 1983 at Deutsche Sammlung von Mikroorganismen, Göttingen, West

Germany under the terms of the Budapest Treaty with the deposit No. DSM 2672. It was classified as belonging to Fusarium oxysporum.

Broth containing protease of the invention may be obtained by cultivating said strain according to principles known in the art, e.g. US 3,652,399 (Takeda) or to an example of this specification.

The culture broth contains at least two proteases (I and II, with II being the protease of the invention) as well as other proteins. Separation can be made by affinity chromatography on a bacitracin-sepharose column, but it was found that a column of soy bean trypsin inhibitor-sepharose (STI-sepharose) has a better capacity.

By use of 0.05M boric acid, pH 6.5 as buffer, proteases I and II will be bound, while other protein is eluted. The unwanted protease I can then be eluted by 0.25M NaCl in the same buffer, by 0.1M NaCl or by 0.05 M boric acid at pH 4-5 or below. Protease II (protease of the invention) can finally be eluted by 0.05M acetic acid, pH 2.8.

SDS-PAGE and isoelectric focusing (IEF) in the presence of marker proteins are convenient methods for molecular weight (MW) and isoelectric point (pI) determinations, respectively. According to these methods, protease of the invention has MW of about 27 kD and pI of 9-10. Protease I (described above) has MW of about 30 kD and pI 9-10, and prior-art Fusarium protease produced by cultivation of strain S-19-5 according to US 3,652,399 contains a single component with MW of about 32 kD by the above method.

The pH and temperature dependence of activity is shown in fig. 2 and 3, where protease I is also shown for comparison. The curves are based on the CPU method (described below), except that temperature or pH is varied. As shown in the figures, protease of the

invention has temperature optimum around 45°C (30 minutes reaction at pH 9.5), and pH optimum at 8.5-11.0 (30 minutes reaction at 25°C) with nearly constant activity in that pH range.

The pH and temperature curves were also measured in solutions of a built liquid detergent and of a powder detergent with perborate and TAED (bleach activator). These curves were nearly identical to those without detergent shown in figs. 2 and 3.

The protease is inhibited by inhibitors characteristic for serine proteases, such as PMSF.

To illustrate the specificity, oxidized B-chain of bovine insulin was hydrolyzed by protease (1.38 CPU/l, 15 min), and the hydrolysis products were analyzed by reverse-phase liquid chromatography (5 micron silica coated with C-18 hydrocarbon, gradient elution). Fig. 4 shows the results with protease of the invention, fig. 5 with component I from DSM 2672, and fig. 6-7 with prior-art Fusarium proteases according to US 3,652,399 (Fusarium sp. S-19-5 and F. oxysporum f. batatas IFO 4468, respectively).

The chromatograms show a striking difference. That of the protease of the invention has only two major peaks and appears to be similar to the chromatogram obtained with trypsin, which is known to hydrolyze two peptide bonds (Arg(22)-Gly(23) and Lys(29)-Ala(30)). In contrast, the other Fusarium proteases hydrolyze a multitude of bonds in this substrate, resulting in more than 10 peaks of comparable size.

The substrate specificity is also illustrated by the action on two synthetic substrates. Protease of the invention hydrolyzes Bz-Arg-pNA but not Suc-AAPF-pNA. Protease I, in contrast, hydrolyzes only the latter.

Monospecific antisera against purified protease may be raised for example by immunizing rabbits according to the regimen described by N. Axelsen et al. in: A

manual of Quantitative Immunoelectrophoresis, Blackwell Scientific Publications, 1973, chapter 23. Purified immunoglobulins may be obtained from the antisera, for example by salt precipitation ($(NH_4)_2SO_4$), followed by dialysis and ion exchange chromatography, e.g. on DEAE-Sephadex.

Immunochemical characterization of proteins may be conducted either by Ouchterlony double-diffusion analysis (O. Ouchterlony in: Handbook of Experimental Immunology (D.M. Weir, Ed.), Blackwell Scientific Publications, 1967, pp. 655-706), by crossed immunoelectrophoresis (N. Axelsen et al., supra, chapters 3 and 4), or by rocket immunoelectrophoresis (N. Axelsen et al., supra, Chapter 2).

The protease of the invention shows immunochemical non-identity to protease I from DSM 2672 and to S-19-5 prior-art Fusarium protease.

Detergent composition

The detergent compositions of the invention comprise surfactant which may be of the anionic, non-ionic, cationic, amphoteric or zwitterionic type, or a mixture of these. Typical examples of anionic surfactant are linear alkyl benzene sulfonate (LAS), alpha olefin sulfonate (AOS), alcohol ethoxy sulfate (AES) and natural soap of alkali metals.

Detergent according to the invention may contain other detergent ingredients known in the art, such as builders, bleaching agents, bleach activators, anti-corrosion agents, sequestering agents, anti-soil redeposition agents, perfumes, stabilizers for the enzymes and bleaching agents and so on.

The detergent compositions of the invention can be formulated in any convenient form, such as powders, liquids, etc. The protease may be stabilized in a liquid detergent by inclusion of enzyme stabilizers, e.g. those mentioned above.

Detergents usually have a pH in solution of 7-12, especially 8-10.5. Specific protease with activity at this pH is preferred.

The detergent of the invention may contain one or more other detergent enzymes in addition to protease of the invention. Examples are lipase, amylase and cellulase. It is known that when combining a protease with another enzyme in a detergent, the other enzyme becomes liable to digestion and deactivation by the protease in the detergent solution (see e.g. EP 205,208 (Unilever). In this connection, the high substrate specificity of the protease of the invention makes it more compatible with other enzymes. The two (or more) enzymes may be added separately or in the form of a combined additive.

Detergent additive

The detergent additive of the invention is in the form of a non-dusting granulate, a stabilized liquid or a protected enzyme.

Non-dusting granulates may be produced e.g. according to NL 167,993 (Novo), US 4,106,991 (Novo) or US 4,661,452 (Novo) and may optionally be coated according to principles known in the art.

A liquid protease preparation may be stabilized e.g. by adding propylene glycol, other polyols, sugars, sugar alcohols, lactic acid, boric acid. Other enzyme stabilizers are known in the art.

Protected enzyme may be produced according to EP 238,216 (Novo, Albright & Wilson).

The detergent additive of the invention may contain one or more other detergent enzymes, e.g. lipase, cellulase or amylase. In the case of a granulate, the enzymes may be mixed before or after granulation.

Protease activity (CPU)

Proteolytic activity is determined with casein as the substrate. One Casein Protease Unit (CPU) is defined as the amount of enzyme liberating 1 millimole of primary amino groups (determined by comparison with a serine standard) per minute under standard conditions, i.e. incubation for 30 minutes at 25°C and pH 9.5.

A 2% (w/v) solution of casein (Hammarsten, supplied by Merck A.G., West Germany) is prepared with the Universal Buffer described by Britton and Robinson (Journ.Chem.Soc. 1931, p. 1451), adjusted to pH 9.5.

Two ml of substrate solution is preincubated in a water bath for 10 minutes at 25°C. 1 ml of enzyme solution containing b g/ml of enzyme preparation, corresponding to about 0.2 - 0.3 CPU/ml of Britton-Robinson buffer (pH 9.5), is added. After 30 minutes of incubation at 25°C the reaction is terminated by the addition of a stopping agent (5 ml of a solution containing trichloroacetic acid (17.9 g), sodium acetate (29.9 g), and acetic acid (19.8 g), filled up to 500 ml with deionized water). A blank is prepared in the same manner as the test solution, except that the stopping agent is added prior to the enzyme solution.

The reaction mixtures are kept for 20 minutes in the water bath, whereupon they are filtered through Whatman® 42 paper filters.

Primary amino groups are determined by their colour development with o-phthaldialdehyde (OPA).

Disodium tetraborate decahydrate (7.62 g) and sodium dodecylsulfate (2.0 g) is dissolved in 150 ml of water. OPA (160 mg) dissolved in 4 ml of methanol is then added together with 400 µl of beta-mercaptoethanol, whereafter the solution is made up to 200 ml with water.

To the OPA reagent (3 ml) is added 400 µl of the above-mentioned filtrates with mixing. The optical density (OD) at 340 nm is measured after about 5 minutes.

The OPA test is also performed with a serine standard containing 10 mg of serine in 100 ml of Britton-Robinson buffer (pH 9.5). The buffer is used as a blank.

The protease activity is calculated from the optical density measurements by means of the following formula:

$$\text{CPU/ml of enzyme solution} = \frac{(OD_t - OD_b) \times C_{ser} \times Q}{(OD_{ser} - OD_B) \times MW_{ser} \times t_i}$$

CPU/g of enzyme preparation = CPU/ml: b

wherein OD_t, OD_b, OD_{ser} and OD_B is the optical density of the test solution, blank, serine standard, and buffer, respectively, C_{ser} the concentration of serine in mg/ml in the standard, MW_{ser} the molecular weight of serine. Q is the dilution factor (in this instance equal to 8) for the enzyme solution, and t_i is the incubation time in minutes.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows the elution chromatogram of culture broth from strain DSM 2672 on a STI-Sepharose column. Details are given in Example 2.

Figs. 2 and 3 show the pH-activity and temperature activity curves, respectively, of the two proteases from DSM 2672, viz. protease II (protease of the invention) and protease I for comparison.

Figs. 4 - 7 show reverse-phase chromatograms of hydrolysis products of oxidized B-chain of bovine insulin with Fusarium proteases. Fig. 4 shows hydrolysis with protease of the invention, fig. 5 with protease I from DSM 2672, fig. 6 with protease from S-19-5, and fig. 7 with protease from F. oxysporum f. batatas (IFO 4468), the two latter prepared according to US 3,652,399.

Figs. 8 - 13 show the results of the washing trials of Examples 3 - 8, respectively.

Fig. 14 shows the results of the washing tests in Example 10.

EXAMPLES

EXAMPLE 1

Fermentation of F. oxysporum DSM 2672

A seed fermenter with the below medium was inoculated with DSM 2672 and fermented for 30-35 hours with aeration. It was then used for seeding a 10 times larger main fermenter with the same medium and fermented for 114 hours with aeration and with continuous dosing of additional substrate.

Medium composition (w/v%):

Soy meal	5.0
glucose	5.0
KH_2PO_4	2.0
K_2HPO_4	2.0
$CaCl_2.2H_2O$	0.02
$MgSO_4.7H_2O$	0.02
Soy bean oil	0.5
Pluronic® (ml/l)	0.033

Substrate dosed: 45% w/v glucose
dosing rate: 0.28% vol/vol/hour

The protease activity of the broth was 2.84 AU/l (AU indicates protease activity in Anson Units, see US 3,723,250, col. 8).

Crude protease was recovered from the culture broth by addition of ammonium sulfate (salting out), filtration, redissolution of filter cake, purification by ion exhange followed by bentonite, and finally drying.

EXAMPLE 2

Separation of proteases

Crude protease obtained as in Example 1 (activity 15 CPU/g) was dissolved in buffer (0.05M boric acid, pH 6.5). After decolorization by adsorption on DEAE-Sephadex, the OD was 1.2 at 280 nm. 15 ml of this solution with a total protein content of 18 mg (calculated from OD, 280 nm), was separated by affinity chromatography.

The gel was soy bean trypsin inhibitor-agarose (STI-agarose), the bed volume was 10 ml, and the column diameter was 2.5 cm. The above buffer was used, with a flow rate of 0.5 ml/min, and fractions of 4.5 ml each were collected. For each fraction, OD 280 nm was measured, as well as activity towards Bz-DL-Arg-pNA and Suc-AAFF-pNA. The elution chromatogram is shown in fig. 1. The eluent used at each stage is also indicated in the figure.

As shown in fig. 1, unadsorbed material was first eluted with buffer, then a protease peak (termed I) was eluted by 0.25M NaCl in buffer. As shown, increase to 0.5M NaCl and application of 0.1M acetate, pH 4.0 eluted no further material. A second protease peak (termed II) was eluted by 0.05M acetic acid, pH 2.8.

Distribution of protein:

Injected		18 mg
Eluted		
Not adsorbed	7.45 mg (41%)	
Component I	6.55 mg (36%)	
Component II	4.03 mg (22%)	
Total		18 mg

Approx. 64% of the protein remained in solution after the initial purification by DEAE, so the protein recovery in relation to the protease concentrate was as follows:

Component I	23% of protein (62% of protease)
Component II	14% of protein (38% of protease)

EXAMPLES 3 - 8

Washing trials

The following detergent solutions (in g/l) were used. Nos. I-III represent powder detergents, and No. IV a liquid built detergent.

	I	II	III
LAS	0.4	0.4	0.4
AE	0.15	0.15	0.15
Soap	0.15	0.15	0.15
Sodium tripolyphosphate	1.75	-	1.75
Sodium silicate	0.4	0.4	0.4
Carboxy methyl cellulose	0.05	0.05	0.05
EDTA	0.01	0.01	0.01
Sodium sulfate	2.1	2.1	2.1
Sodium perborate	-	-	1.0
TAED	-	-	0.1
Zeolite A	-	1.25	-
NTA	-	0.5	-
Sodium carbonate	-	0.5	-
NaOH to pH:	9.5	10.0	9.5

	IV
AES	0.23
AE	0.23
Oleic acid	0.075
Triethanolamine	0.15
Ethanol	0.03
Propylene glycol	0.15
DTPA	0.008
Disodium citrate, $2H_2O$	0.17
$CaCl_2$, $2H_2O$	0.015
NaOH to pH:	8.0

LAS is linear alkyl benzene sulfonate (Nansa 80S, product of Albright & Wilson, UK), AE is alcohol ethoxylate (Berol 065, product of Berol Kemi AB, Sweden), EDTA is ethylene diamine tetra-acetic acid, TAED is N,N,N,N-tetra-acetyl ethylene diamine, NTA is nitrilotriacetic acid, AES is alcohol ethoxy sulfate (Dobanol 25-3S, product of Shell Chemicals), DTPA is diethylene triamine penta-acetic acid tri-sodium mono-calcium salt.

Soiled spinach swatches were made on a Mathis Washing and Drying Unit (Werner Mathis AG, Switzerland) in continuous operation, whereby cotton textile passes through spinach juice, is squeezed between two rollers and is then blown dry with 30°C air (thermostated). The swatches were aged for 3 weeks at 20°C, and were then kept at -18°C until use.

Swatches with mixed soiling were made by immersing cotton in the below mixture; squeezing between rollers; drying and aging for 2 days in air at 20°C.

Olive oil	14.4	weight %
Stearic acid	1.8	"
Monoglyceride	1.8	"
Gelatine	0.9	"
Deionized water	79.3	"
Carbon black	0.2	"
China clay	1.4	"
Indian ink	0.2	"

Washing tests were made in a Terg-O-Tometer (Jay C. Harris: Detergency Evaluation and Testing, Interscience Publishers Ltd. (1954), pp. 60-61) with 7 swatches (7 x 7 cm) and 700 ml detergent solution in each beaker. Conditions were 25°C, 10 min, 100 rpm. After rinsing and drying, reflectance (R) of the swatches at 460 nm was measured. The washing performance is expressed as delta R = $R-R_o$, where R_o is the measurement without enzyme.

Protease of the invention (prepared as in Example 2) was compared to component I (prepared as in Example 2) and to Savinase® (an alkaline Bacillus protease, product of Novo Industri A/S, Denmark).

Results are given as R_o and delta R versus protease type and dosage (in CPU/l). The below results are also shown in figures as indicated:

Example 3 (fig. 8)

Det. I, spinach soiling

	CPU/l				
Protease	0	0.025	0.05	0.075	0.1
	R_o	delta R	delta R	delta R	delta R
Invention		8.3	11.2	13.5	15.3
Comp. I	42.2	2.6	7.0	10.3	11.5
Savinase		2.5	5.1	8.3	11.3

Example 4 (fig. 9)

Det. II, spinach soiling

	CPU/l					
Protease	0	0.025	0.05	0.075	0.1	0.2
	R_o	delta R	delta R	delta R	delta R	delta R
Invention		7.9	10.7	12.8	15.5	20.3
Comp. I	43.4	3.7	8.6	11.7	12.6	17.6

Example 5 (fig. 10)

Det. IV, spinach soiling

	CPU/l			
Protease	0	0.025	0.05	0.2
	R_o	delta R	delta R	delta R
Invention		3.5	4.0	7.0
Comp. I	37.7	1.8	2.8	4.7

Example 6 (fig. 11)

Det. I, mixed soiling

	CPU/l			
Protease	0	0.025	0.05	0.1
	R_o	delta R	delta R	delta R
Invention		8.2	11.0	12.8
Comp. I	21.0	2.3	4.7	8.5

Example 7 (fig. 12)

Det. III, spinach soiling

	CPU/l				
Protease	0 R_0	0.025 delta R	0.05 delta R	0.075 delta R	0.1 delta R
Invention		6.4	10.2	13.5	13.2
Comp. I	40.5	3.6	6.4	8.5	10.9
Savinase		2.2	3.6		9.7

Example 8 (fig. 13)

Det. II, mixed soiling

	CPU/l				
Protease	0 R_0	0.022 delta R	0.044 delta R	0.066 delta R	0.088 delta R
Invention		18.5	20.7	23.1	26.6
Comp. I	21.5	5.2	8.8	11.0	13.9

The detergent formulations tested cover pH 8-10, with various builders (phosphate and non-phosphate) with and without perborate, and with anionic and non-ionic surfactant. Thus, a wide range of typical formulations for liquid and powder detergents have been covered. At all these conditions, protease of the invention showed superior washing effect on the basis of CPU activity.

EXAMPLE 9

Washing trials with protease mixtures

Detergent No. I of examples 3-8 was used. Swatches and washing conditions were as in example 3-8. The protease of the invention, component I and various mixtures of these were added at total dosage up to 0.10 CPU/l.

Protease ratio	CPU/l			
Invention : comp. I	0 R_o	0.01 delta R	0.05 delta R	0.10 delta R
0 : 100	45.1	1.8	7.1	9.7
50 : 50	44.7	1.7	9.1	11.5
75 : 25	44.7	2.1	8.8	11.2
90 : 10	45.1	2.0	9.1	11.2
99 : 1	45.1	2.2	9.5	12.2
100 : 0	44.7	2.2	11.0	14.3

It is seen that increasing purity of the specific protease gives increased washing performance.

EXAMPLE 10

Washing trial with Asp-N specific protease

The following detergent formulation was used:

LAS	0.40 g/l
AE	0.15 g/l
Soap	0.15 g/l
Na_2SO_4	2.00 g/l

The same chemicals as described in examples 3 - 8 were used.

The detergent was dissolved in 10 mM NH_4HCO_3 prepared from 9°GH water, and pH was adjusted to 8.0.

Soiled spinach swatches were prepared like in examples 3 - 8

Washing tests were made in 150 ml glass beakers in a thermostat water bath with magnetic stirring with 6 swatches (2.2 x 2.2 cm each) and 60 ml detergent solution in each beaker. Conditions were 35°C, 90 min.

As another example of specific proteases, the specific protease Asp-N endoproteinase commercially available from Boehringer (cat. No. 1054 589) is compared to the unspecific Alcalase® and Subtilisin Novo (both Bacillus proteases, products of Novo Industri A/S, Denmark).

The proteases are dosed on equal amounts of enzyme protein, 0.6 and 1.2 mg/l.

After rinsing and drying, reflectance (% R) of the swatches were measured according to examples 3 - 8.

Results are given as delta R versus protease type and dosage in figure 14.

EXAMPLE 11

This example demonstrates how adsorption to cotton of hydrolysis products of hemoglobin varies with the type of protease used to hydrolyze the hemoglobin. It is shown that hydrolysis products formed by a specific

protease (Trypsin) are adsorbed much less than those obtained with an unspecific protease (Subtilisin Carlsberg).

The method was as follows:

A 0.05% (w/v) solution of hemoglobin in Britton and Robinson I buffer, pH 9.0, is hydrolyzed by 0.3 CPU/l of a protease. After 30 minutes at 25°C a cotton swatch (circular, 5 cm in diameter) is placed in the reaction mixture and it is boiled for 10 minutes. Then the swatch is removed, rinsed under running water, soaked in deionized water for 30 minutes and rinsed again under running water. After air-drying, reflectance (R) of the swatches at 460 nm is measured. The degree of adsorption is expressed as delta $R = R-R_o$, where R_o is the reflectance obtained without enzyme, i.e. with unhydrolyzed hemoglobin.

The specific protease Trypsin and the unspecific protease Subtilisin Carlsberg were tested, separately and in combination. The results are shown in the following table:

Total activity 0.3 CPU/l		
% Trypsin	% Carlsberg	Delta R
0	100	-6.8
100	0	-0.1
90	10	-3.8
50	50	-5.0

A larger negative value of delta R indicates a darker swatch, i.e. more degradation products adsorbed.

The results show that the unspecific protease has a negative effect, i.e. leads to degradation products that are more easily adsorbed, whereas the specific protease has virtually no such effect. It is also seen that incorporation of even a minor proportion of the unspecific protease leads to significantly more adsorption results than with the pure specific protease.

International Application No: PCT/ /

MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 5, line 2 of the description [1]

A. IDENTIFICATION OF DEPOSIT [2]

Further deposits are identified on an additional sheet ☐ [3]

Name of depositary institution [4]

Deutsche Sammlung von Mikroorganismen

Address of depositary institution (including postal code and country) [4]

Mascheroder Weg 16, D-3300 Braunschweig, West Germany

Date of deposit [5]	Accession Number [6]
6 June 1983	DSM 2672

B. ADDITIONAL INDICATIONS [7] (leave blank if not applicable). This information is continued on a separate attached sheet ☐

In respect of those designations in which a European patent is sought, a sample of the deposited micro-organism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample (Rule 28(4) EPC).

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE [8] (if the indications are not for all designated States)

D. SEPARATE FURNISHING OF INDICATIONS [9] (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later [10] (Specify the general nature of the indications e.g., "Accession Number of Deposit")

E. ☒ This sheet was received with the international application when filed (to be checked by the receiving Office)

Anne Tengen-Grædel
(Authorized Officer)

☐ The date of receipt (from the applicant) by the International Bureau [11]

was

(Authorized Officer)

CLAIMS

1. A detergent composition comprising protease, characterized in that the protease activity affecting detergency is essentially provided by a protease with specificity for peptide bonds adjacent to only one or two specific amino acids.

2. A detergent composition according to Claim 1, being essentially devoid of other proteases.

3. A detergent composition according to Claims 1 or 2, wherein the protease is an animal or microbial protease or is produced by cultivation of a transformed host organism, containing a gene encoding for and expressing an animal or microbial protease.

4. A detergent composition according to any of Claims 1 - 3, wherein the protease has trypsin-like specificity.

5. A detergent composition according to Claim 4, wherein the protease is trypsin.

6. A detergent composition according to Claim 4, wherein the protease is characterized by:

a) being a serine protease
b) the ability to hydrolyze the oxidized B-chain of bovine insulin so that a chromatogram shows only two major hydrolysis products.
c) showing immunochemical identity to a protease obtained by cultivation of Fusarium sp. DSM 2672
d) an isoelectric point of about 9-10
d) ability to hydrolyze Bz-Arg-pNA
e) essentially no hydrolysis o Suc-AAPF-pNA

f) essentially the same activity towards casein at pH 9 and pH 11

7. Detergent additive comprising protease in the form of a non-dusting granulate, stablized liquid or a protected enzyme, characterized as in any of Claims 1-6.

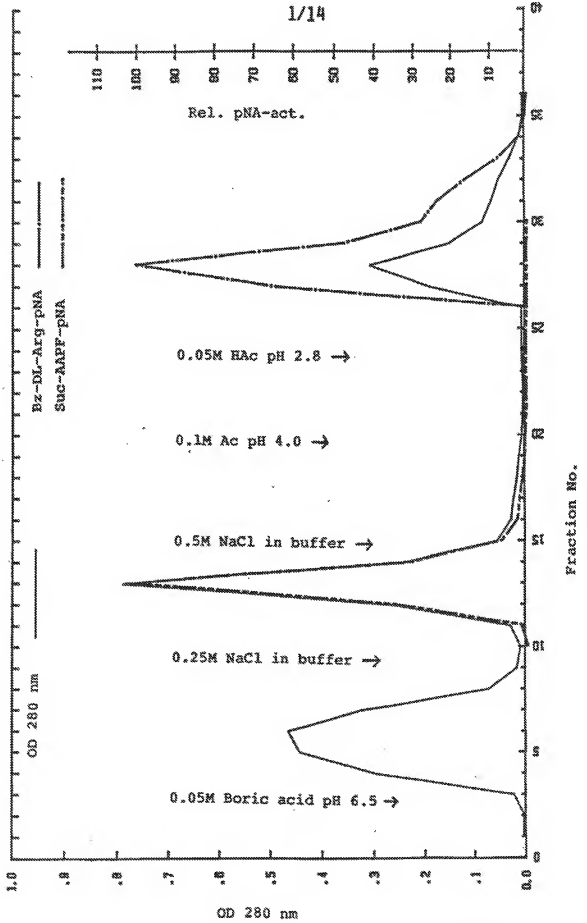

Rel. pNA-act.
Bz-DL-Arg-pNA
Suc-AAPF-pNA
OD 280 nm
0.05M HAc pH 2.8 →
0.1M Ac pH 4.0 →
0.5M NaCl in buffer →
0.25M NaCl in buffer →
0.05M Boric acid pH 6.5 →
Fraction No.
OD 280 nm

FIG. 1

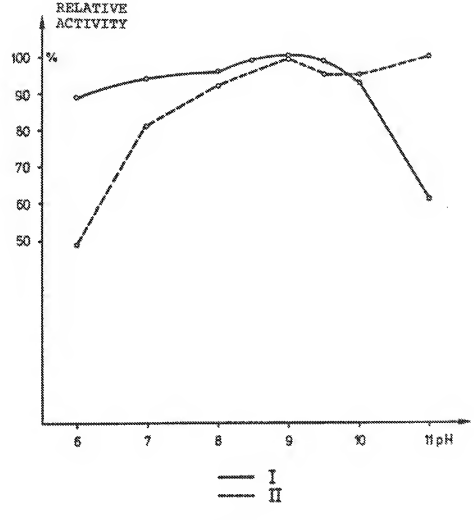


Fig.2

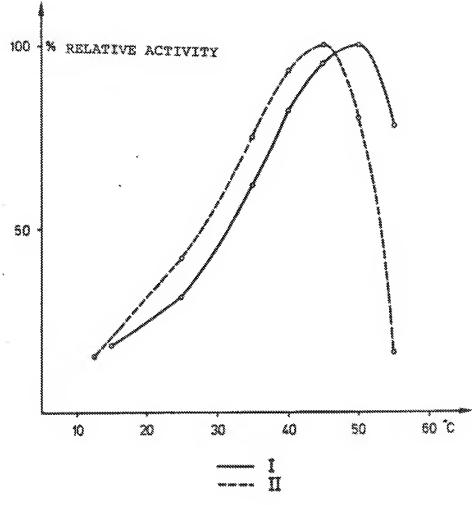


Fig.3

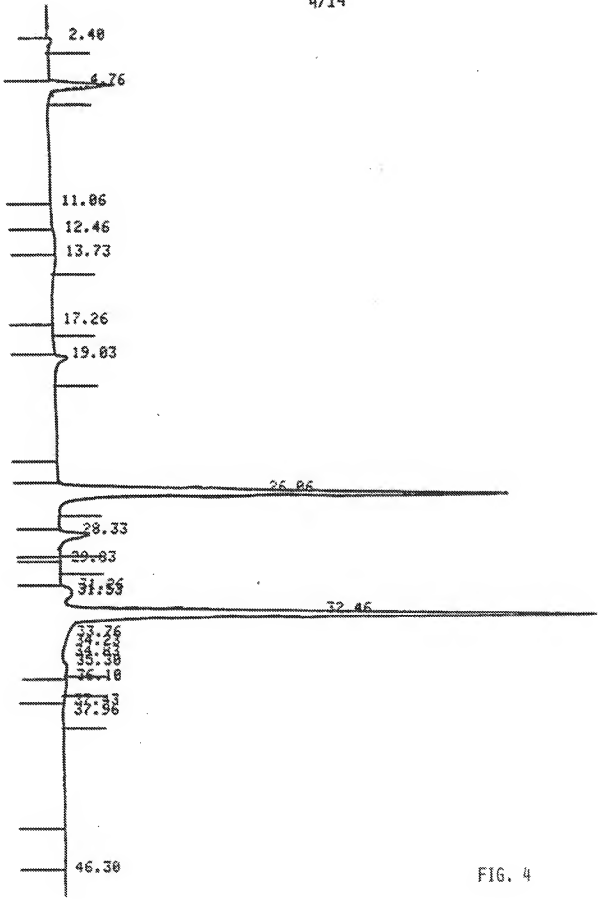

2.40
4.76
11.86
12.46
13.73
17.26
19.03
26.06
28.33
29.03
31.56
32.46
34.26
34.53
35.30
36.10
37.13
37.36
46.30

FIG. 4

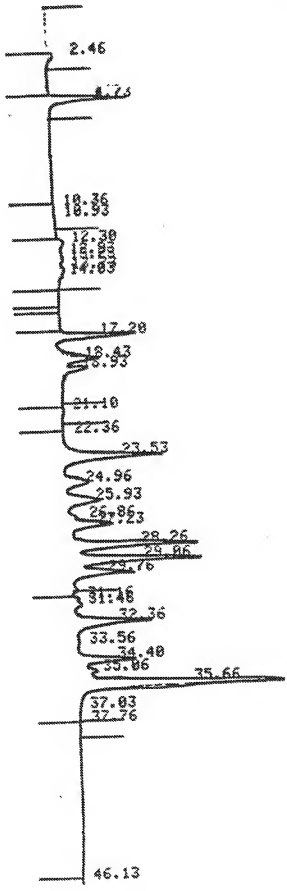

2.46
18.36
18.93
16.30
16.29
14.03
17.20
18.43
21.10
22.36
23.53
24.96
25.93
26.86
27.23
28.26
29.06
31.46
32.36
33.56
34.40
35.66
37.03
37.76
46.13

FIG. 5

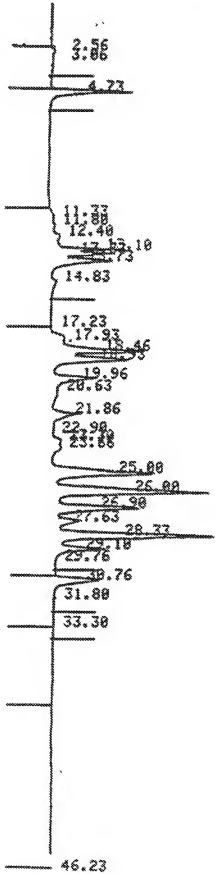

2.56
3.66
4.23
11.37
11.88
12.40
14.83
17.23
17.93
19.96
20.63
21.86
22.90
23.66
25.00
26.00
26.90
27.63
28.37
29.18
29.76
30.76
31.80
33.30
46.23

FIG. 6

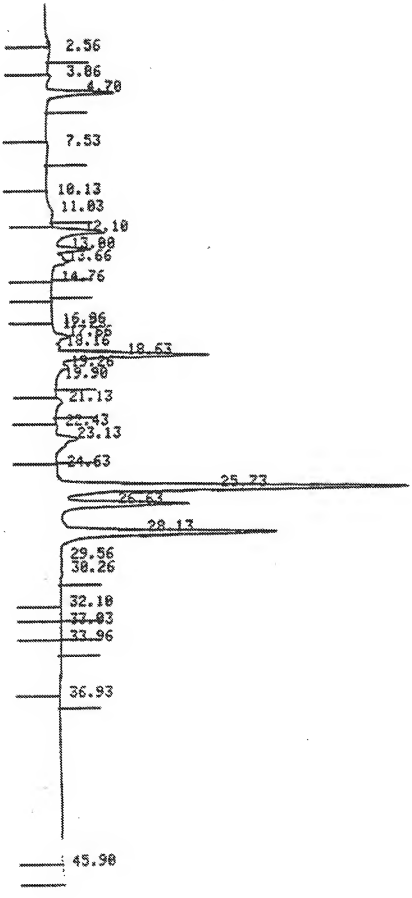

2.56
3.86
4.70
7.53
10.13
11.03
12.10
13.00
13.66
14.76
16.96
17.56
18.16
18.63
19.26
19.90
21.13
22.43
23.13
24.63
25.23
26.63
28.13
29.56
30.26
32.10
32.83
33.96
36.93
45.90

FIG. 7

Fig. 8

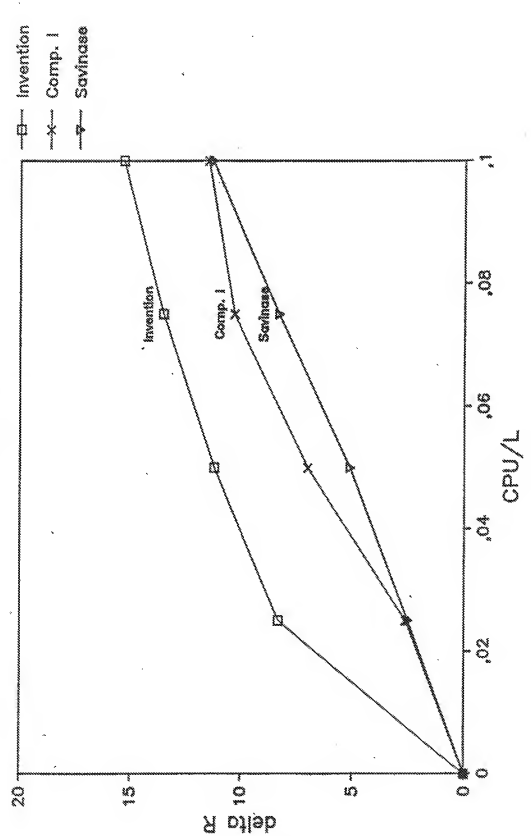

Invention
Comp. I
Savinase
delta R
20
15
10
5
0
0
,02
,04
,06
,08
,1
CPU/L
Invention
Comp. I
Savinase

Fig. 9

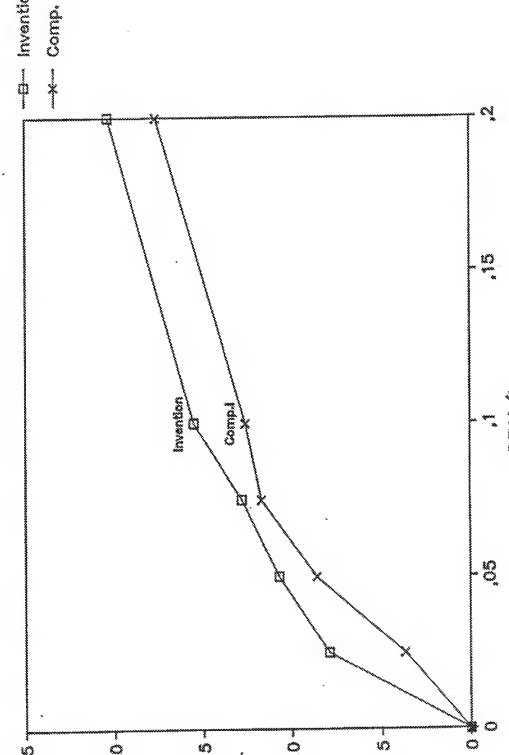

Invention
Comp. I
delta R
25
20
15
10
5
0
0
,05
,1
,15
,2
CPU/L
Invention
Comp.I

Fig. 10

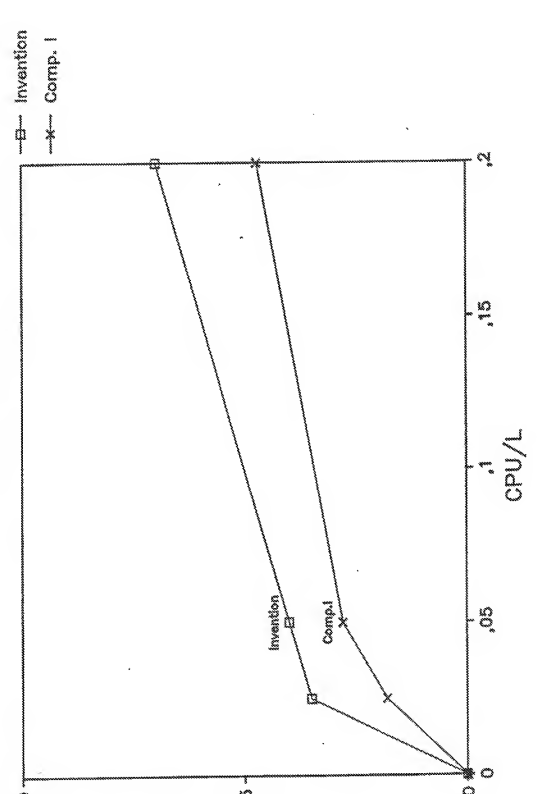


Fig. 11

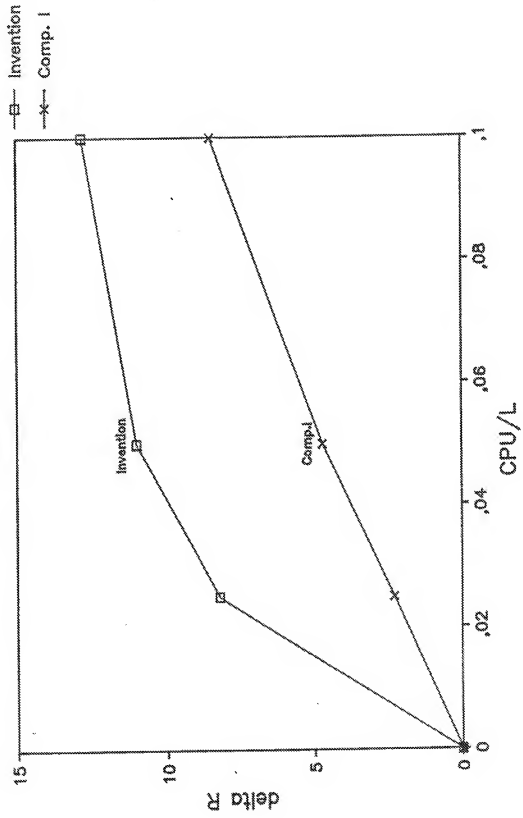

Invention
Comp. 1
delta R
15
10
5
0
0
,02
,04
,06
,08
,1
CPU/L
Invention
Comp.1

Fig. 12

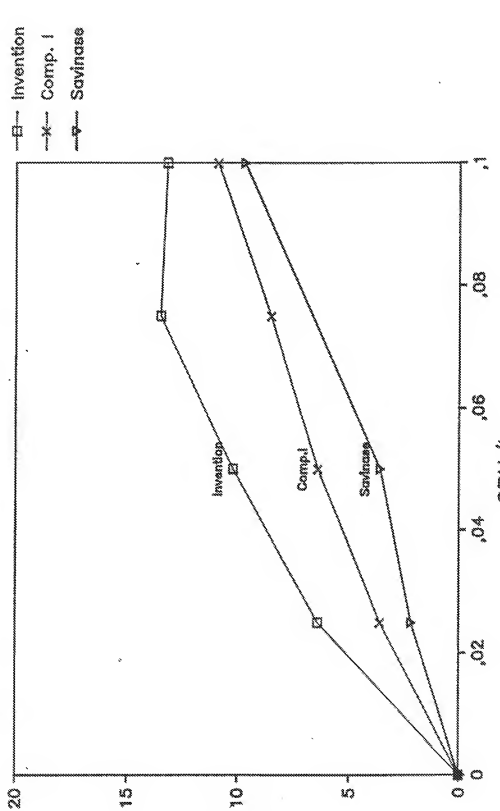
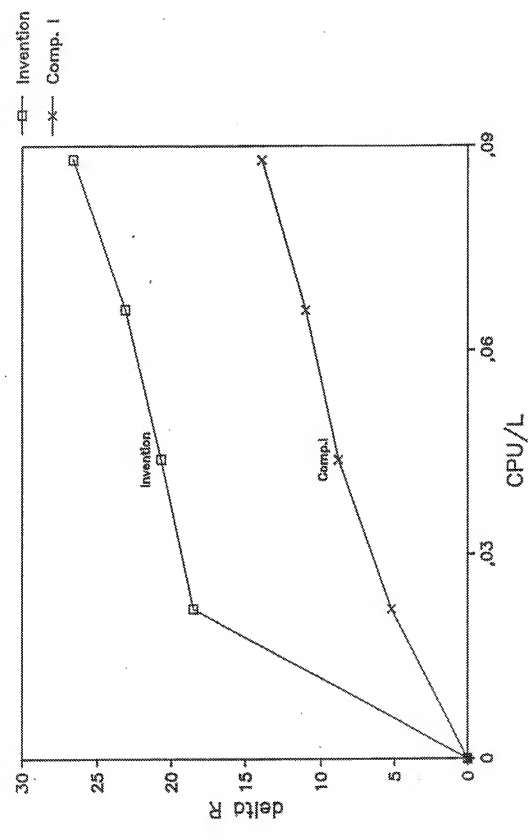

Invention
Comp. I
Savinase
delta R
20
15
10
5
0
0
,02
,04
,06
,08
,1
CPU/L
Invention
Comp.I
Savinase

Fig. 13


Invention
Comp. 1
delta R
30
25
20
15
10
5
0
0
,03
,06
,09
CPU/L
Invention
Comp.1

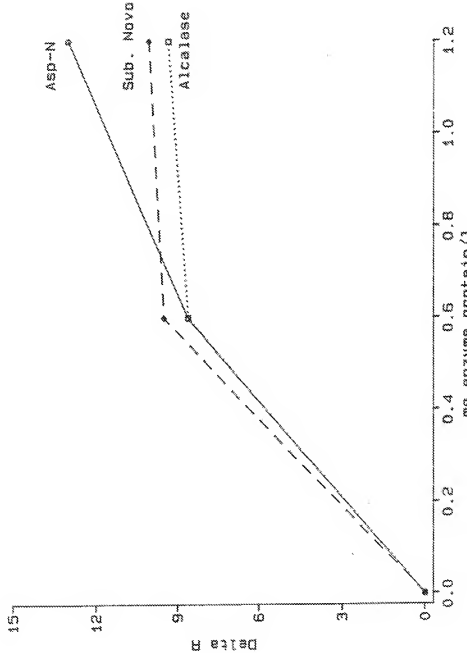

Asp-N
Sub. Novo
Alcalase
Delta B
15
12
9
6
3
0
0.0
0.2
0.4
0.6
0.8
1.0
1.2
mg enzyme protein/l

FIG. 14

INTERNATIONAL SEARCH REPORT

International Application No PCT/DK89/00001

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

C 11 D 3/386

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC 4	C 11 D 3/386, 7/42; C 12 N 9/58, 9/60
US Cl	252:174.12

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

SE, NO, DK, FI classes as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	US, A, 3 652 399 (M. ISONO et al.) 28 March 1972 The whole document; see especially the abstract; column 1, lines 3-5; column 3, lines 60 - column 4, line 11	1-3, 7
A	GB, A, 1 356 130 (THE PROCTOR & GAMBLE COMPANY) 12 June 1974 The whole document, see especially the claims	1-3, 7
A	US, A, 4 264 738 (V.M. STEPANOV et al.) 28 April 1981 The whole document, see especially column 1, lines 6-11 and column 2, lines 52-56	1-7
A	EP, A2, 0 199 404 (THE PROCTER & GAMBLE COMPANY) 29 October 1986 The abstract and claim 1	1

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle of theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
1989-04-10	1989 -04- 12
International Searching Authority	**Signature of Authorized Officer**
Swedish Patent Office	Dagmar Järvman

Form PCT/ISA/210 (second sheet) (January 1985)